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Circulating miRNAs in patients with Barrett's esophagus, high-grade dysplasia and esophageal adenocarcinoma

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Background: Diagnostic screening of premalignant esophageal lesions is hampered by the absence of biomarkers indicative of metaplastic and/or malignant transformation. The aim of this exploratory study was to investigate the potential use of miRNAs as biomarkers capable of identifying patients with (pre)malignant lesions: Barrett's esophagus (BE) metaplasia, high-grade dysplasia (HGD) or esophageal adenocarcinoma (EAC).

Methods: A total of 69 patients were included in the study. Six serum samples from each of four study groups, i.e., patients with normal squamous epithelium (SE), BE, HGD and EAC, were profiled using the Nanostring miRNA analysis platform. Differential miRNA expression patterns then were validated in 69 patient samples using qRT-PCR.

Results: miRNA expression profiling revealed seven miRNAs with a 2-fold change in expression level. Validation by qRT-PCR confirmed that serum miR-320e levels were significantly decreased in the BE group compared to the SE ($P \leq 0.001$, AUC 0.790) and HGD groups ($P \leq 0.005$, AUC 0.786). Serum miR-199a-3p levels were significantly decreased in the BE group compared to the SE group ($P \leq 0.001$), area under the curve (AUC) of 0.813.

Conclusions: The results of this study suggest that decreased serum miRNA levels of miR-199a-3p and miR-320e could help to identify patients with BE and HGD.

Keywords: Screening; biomarkers; cancer

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Introduction

Esophageal adenocarcinoma (EAC) is a deadly disease with a rising incidence. While patients with early EAC lesions have a 5-year survival of 78%, this drops to around 47% in patients with locally advanced disease (1,2). Early detection significantly improves survival outcome (3,4), but the majority of EAC cases are diagnosed at an advanced stage.

EAC is thought to develop through a metaplasia-dysplasia-carcinoma sequence. The metaplastic lesion is known as Barrett's esophagus (BE). Patients with BE are advised to undergo regular endoscopic surveillance to detect early malignant transformation. Persistent gastro-esophageal reflux disease (GERD) is the main risk factor for BE development. Currently, BE is diagnosed by histologic examination of endoscopic biopsies, but the low absolute incidence of BE

in GERD patients and the invasive nature of the endoscopy preclude its use as a screening tool in patients at risk for BE development (5,6). A screening strategy that at least partially mitigates these drawbacks could contribute to an early detection of BE, allowing surveillance and/or early treatment and so decrease morbidity and mortality.

Currently, no blood-based biomarkers are available for BE, high-grade dysplasia (HGD) or EAC. miRNAs are a class of small non-coding RNAs that act as negative regulators of gene expression by inhibition of mRNA translation. miRNAs are highly tissue-specific and various studies indicate that alterations in miRNA expression have key roles in carcinogenesis (7,8). Moreover, miRNAs are stable in the circulation and alterations in circulating miRNA levels have been detected in various solid malignancies (9). Circulating miRNAs might thus be attractive blood-based biomarkers of BE and EAC. Several studies showed significant differences in tissue miRNA expression patterns between normal squamous epithelium (SE), BE and EAC (10-13). However, it is currently not known whether these differences are also reflected in the circulating miRNA profile. The aim of this study was to investigate whether circulating miRNAs could be used to differentiate between patients with normal SE and patients with (pre)malignant changes in the esophageal epithelium (BE, HGD and/or EAC).

Methods

Patients and samples

Patients scheduled to undergo an esophagogastroduodenoscopy were prospectively recruited from 2012 up to and including 2014 into one of four study groups: the control group consisting of patients without endoscopic esophageal abnormalities (SE group), a group consisting of patients with BE without dysplasia, a group consisting of patients with columnar HGD or early stage (\leq T1a) EAC (HGD group) and a group consisting of patients with advanced EAC (stage \geq T1b, EAC group). For each participant, the diagnosis was confirmed based on endoscopic appearance, pathological biopsy analysis and imaging [endoscopic ultrasound (EUS) and/or PET/CT for the HGD and EAC groups]. Each participant was asked about smoking history. General exclusion criteria were a history of previous malignancy in the past 5 years, or known chronic inflammatory disease. The study protocol was approved by the Ethics Committee of the University Medical Center Groningen. Written informed

consent was obtained from all participants. The number of samples included in the study was based on the amount of available material.

Blood collection

Serum samples were collected from each participant. Using venipuncture, approximately 9 mL blood was collected from each participant in a BD vacutainer serum tube (BD, Breda, The Netherlands). The blood was left to clot for one hour at room temperature and serum was isolated by centrifugation and stored at -80°C .

RNA extraction

Serum samples were thawed and filtered through a $0.2\ \mu\text{M}$ filter (Corning Life Sciences, Amsterdam, The Netherlands) to eliminate any residual platelets or cell debris. Total RNA was isolated from 200 μL of serum using the miRNeasy micro kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol, with the addition of Phase Lock Gel Heavy gel tubes (5 Prime, Hilden, Germany). RNA was eluted in 10 μL of RNase-free water and stored at -80°C until further analysis.

Nanostring nCounter miRNA assay

A high-throughput serum miRNA expression profile was generated from a subset of study participants. We selected serum samples of six patients of each study group, resulting in a total of 24 samples. Patients from the different study groups were matched for sex, age and comorbidity, as much as possible given the limited number of patient samples. The number of samples included in the Nanostring assay was based on the number of assays on one Nanostring expression assay ($N=12$). To perform a comprehensive assay of circulating miRNAs in serum we used the nCounter[®] miRNA Expression Assay version 2 which contains probes for 800 mature human miRNAs (Nanostring Technologies, Seattle, USA). An equal volume of RNA eluate was used as input for each sample (5 μL). The Nanostring assay was performed by Nanostring Technologies.

Data analysis

Data analysis was performed using GeneSpring GX version 12.5 software (Agilent Technologies, Santa Clara, USA). Raw Nanostring data were normalized by percentile shift

Table 1 Patient characteristics for each study group

Study group	SE (N=19)	BE (N=27)	HGD (N=17)	EAC (N=6)	P value
Gender (male), n [%]	8 [42]	19 [70]	13 [76]	5 [83]	0.067
Mean age [range]	54.3 [23.0–70.0]	58.7 [25.0–75.0]	65.1 [42.0–82.0]	62.5 [46.0–73.0]	0.086
Smoking, n [%]	4 [21]	5 [19]	5 [29]	4 [67]	0.790
Cardiovascular disease, n [%]	3 [16]	6 [22]	4 [24]	2 [33]	0.834
Autoimmune disease, n [%]	1 [5]	1 [4]	1 [6]	1 [17]	0.691
Diabetes, n [%]	1 [5]	1 [4]	6 [35]	0 [0]	0.004 [†]

[†], after multiple testing correction SE study group vs. HGD study group: P=0.036, BE study group vs. HGD study group: P=0.013. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia; EAC, esophageal adenocarcinoma.

normalization to the 95th percentile. The upper range of expression for negative control oligo's on the Nanostring platform was 30 counts. Therefore, a detection cutoff was defined as at least four out of six samples in at least one of the four study groups having a count number >30. This cutoff yielded expression levels above the background for 62 human miRNAs.

qRT-PCR

miRNA-specific cDNA synthesis was performed using the TaqMan miRNA transcription kit (Life Technologies, Bleiswijk, The Netherlands) in a multiplexed reaction as described previously (14) using specific miRNA reverse transcription primers as provided in the TaqMan microRNA assays: miR-16-5p (ID 000391), miR-144-5p (ID 002148), miR-150-5p (ID 000473), miR-199a-3p (ID 002304), miR-320e (ID 243005_mat), miR-451a (ID 001141) and RNU6B (ID 001093), all from Life Technologies. qRT-PCR was performed using the TaqMan Universal Master Mix II without UNG (Life Technologies) and PCR primers from the above mentioned TaqMan microRNA assays. All qPCR reactions were run in triplicates on the ABI7900HT thermo cycler (Life Technologies). Cycle threshold (Ct) values for all miRNAs were quantified using the Sequence Detection software (version 2.3, Life Technologies). Serum miRNA expression levels were normalized according to the geometric mean of the Ct values of the SE group.

Statistical analysis

Differences in patient characteristics were analyzed using ANOVA with Scheffé's method. Differences in serum miRNA expression between the patient groups

did not follow a normal distribution (evaluated using the D'Agostino-Pearson omnibus test) and were therefore examined using the Kruskal-Wallis test with Dunn's multiple testing correction. For statistical analysis and receiver operating characteristic (ROC) curve construction of the qPCR data the Prism 5.0 statistical package was used (GraphPad Software, San Diego, USA). For all tests, a P value of <0.05 was considered significant.

Results

Patient groups

Patient characteristics are described in *Table 1*. The extended patient characteristics are described in *Table S1*. The only significant difference was observed in the occurrence of diabetes between the SE and HGD and between BE and HGD groups (P=0.036 and P=0.013 respectively).

Identifying differences in circulating miRNA levels

Using the Nanostring platform we identified 62 miRNAs with expression levels above the background (*Figure S1*). Due to the limited sample size we focused on miRNAs with a fold change >2 between the four study groups, which revealed seven differentially expressed miRNAs (*Table 2*). No Taqman assay was available for hsa-miR-4516. The remaining six miRNAs were used for validation by qRT-PCR in an extended patient group consisting of the 24 patients included in the Nanostring array and an additional 45 patients (13 SE, 21 BE and 11 HGD serum samples). Three miRNAs, i.e., miR-150-5p, miR-199a-3p and miR-320e, showed significant differences between the study

Table 2 miRNAs with fold change >2 on the Nanostring assay

Study group	SE vs. BE	SE vs. HGD	SE vs. EAC	BE vs. HGD	BE vs. EAC	HGD vs. EAC
miR-16-5p	–	–	–	–	–	↓
miR-144-3p	–	–	↓	–	↓	↓
miR-150-5p	–	–	↓	–	–	–
miR-199a-3p [†]	–	–	↓	–	–	–
miR-320e	↓	–	–	↑	↑	–
miR-451a	–	–	–	↑	–	↓
miR-4516	–	–	–	↑	–	–

[†], miR-199a-3p has the same sequence as miR-199b-3p. Arrows indicate fold change >2 based on the Nanostring assay. MiR-144-3p was excluded due to technical reasons, and no TaqMan assay was available for miR-4516. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia; EAC, esophageal adenocarcinoma.

groups based on the Kruskal-Wallis test (*Figure 1*). The expression of miR-199a-3p was significantly lower in the BE group compared to the SE group ($P \leq 0.001$) and the expression of miR-320e was significantly lower in the BE and HGD groups compared to the SE group ($P \leq 0.001$ and $P \leq 0.005$ respectively, *Figure 1*). The post-hoc test did not reveal significant differences in miR-150-5p levels between the study groups. No significant differences were observed for the miRNA levels between the four study groups for miR-16-5p and miR-451a (*Figure S2*). miR-144-3p was excluded from further analysis due to insufficient quality of the qRT-PCR assay data.

Potential use of the significantly decreased serum-derived miRNAs as disease biomarkers

Based on the significant differences in expression levels of miR-199a-3p and miR-320e between the SE and the BE/HGD groups, we investigated to what extent the expression of these miRNAs could be used to identify patients with (pre)malignant changes in the esophagus (*Figure 2*). Serum miR-199a-3p levels could discriminate between the SE and BE groups with an area under the curve (AUC) of 0.813 (optimal sensitivity of 85.2% and specificity of 68.4% at relative expression <0.766). Similarly, serum miR-320e levels could discriminate between the SE and BE groups with an AUC of 0.790 (optimal sensitivity of 85.2% and specificity of 63.2% at relative expression <0.844) and between the SE and HGD groups with an AUC of 0.786 (optimal sensitivity of 82.3% and specificity of 62.2% at relative expression <0.838).

Discussion

The lack of easily accessible diagnostic biomarkers of BE and EAC is a significant contributor to the poor overall survival of EAC patients. miRNAs are attractive as potential biomarkers, but there is very limited data regarding circulating miRNAs that could differentiate between patients with normal SE and patients with (pre)malignant changes in the esophageal epithelium. Here, we studied the levels of serum miRNAs in patients at all stages of the metaplasia-dysplasia-carcinoma sequence using the Nanostring platform. We identified seven miRNAs with a fold change >2 between any of the different study groups. Using qRT-PCR in an extended group of patients we were able to validate decreased levels of circulating miR-199a-3p and miR-320e in patients with BE (both miRNAs) and HGD (miR-320e only) compared to the SE group.

miR-199a-3p is a known tumor suppressor miRNA. *In vitro* studies showed that miR-199a-3p reduces cell proliferation and migration through a direct repression of mTOR and c-MET (15-18) and miR-199a-3p tissue levels were decreased in endometrial, thyroid, prostate and hepatocellular carcinoma (15-19). In a previous study miR-199a-3p expression levels were significantly increased in EAC tissue samples compared to SE tissue samples, but not in BE or HGD tissue samples (20). miR-320e was previously found to be increased in EAC tissues compared to BE tissues (21) and high miR-320e tissue levels were associated with an adverse prognosis in colon cancer (22).

It is unclear why miR-199a-3p and miR-320e levels were decreased in the circulation while other studies report an increased expression in malignant tissue. However, similar

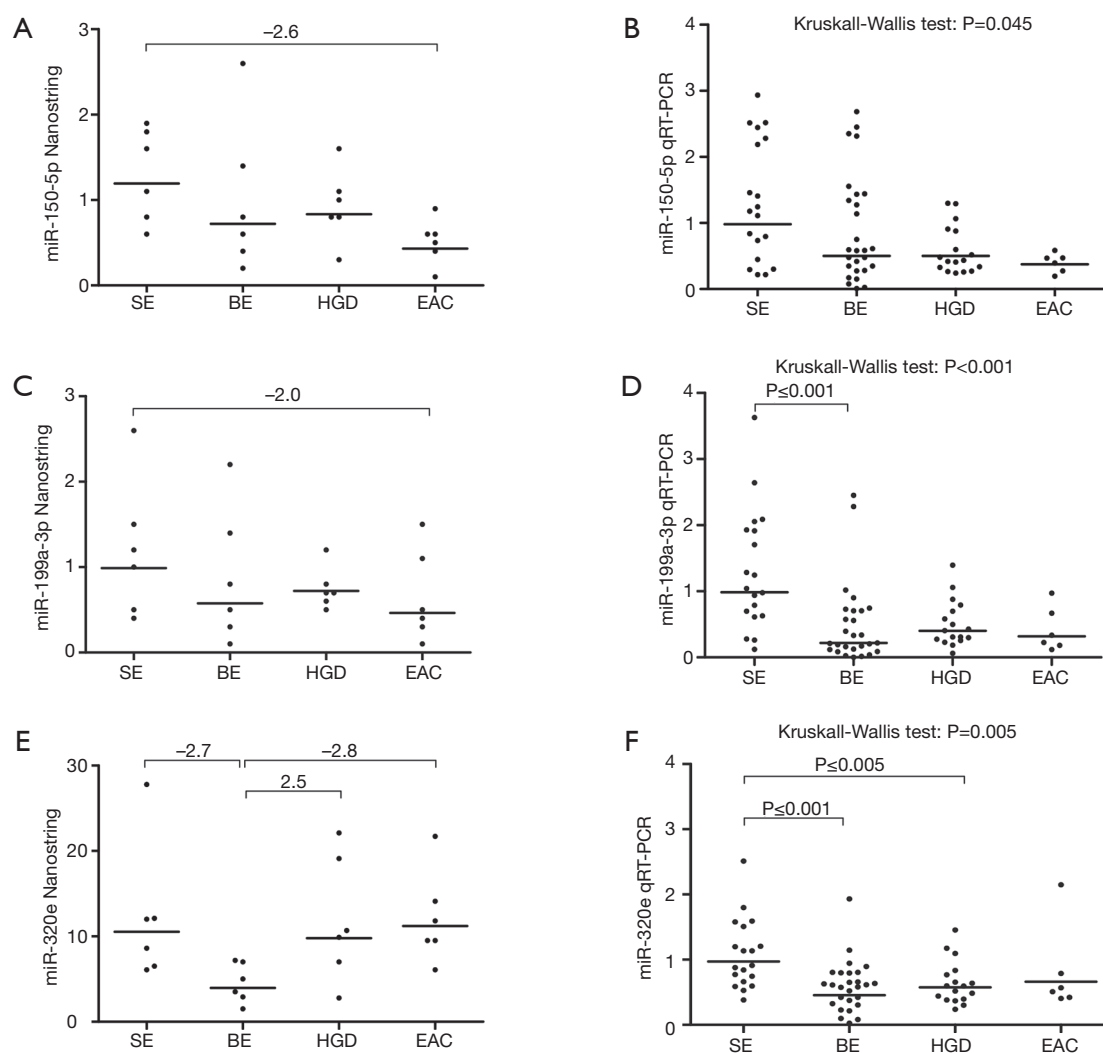


Figure 1 Serum expression of miR-150-5p, miR-199a-3p and miR-320e and validation in the expanded patient cohort. Results of the Nanostring analysis (A,C,E) in the original cohort of 24 serum samples (6 for each study group) and the qRT-PCR validation (B,D,F) in the expanded patient cohort including the same 24 serum samples and 45 additional samples. Shown are the three miRNAs that were significantly differentially expressed in the qRT-PCR validation experiments (Table 2), i.e., miR-150-5p (A,B), miR-199a-3p (C,D) and miR-320e (E,F). No significant differences in miR-150-5p expression were identified between the study groups in the *post-hoc* test (B). Compared to the SE group, serum levels of miR-199a-3p were significantly lower in the BE group (D) and serum levels of miR-320e were significantly lower in the BE and HGD groups (F). Horizontal bars denote geometric mean. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia; EAC, esophageal adenocarcinoma.

findings have been observed previously for other miRNAs (22). Although a mechanistic explanation for increased tissue miRNA levels and a concurrent decrease in the circulating miRNA levels is missing, it seems unlikely that changes in the generally very small affected tissue areas (such as in BE and EAC) could lead to reduced circulating miRNA levels. Furthermore, the origin of serum miRNAs

remains poorly understood. A study by Pritchard *et al.* (23) showed that the majority of previously identified cancer-associated circulating miRNAs, were highly expressed in hematopoietic cells and their levels correlated with blood count levels. This suggested the possibility that changes in blood cell composition and/or integrity (e.g., hemolysis) can affect circulating miRNA levels. Of note, these miRNAs did

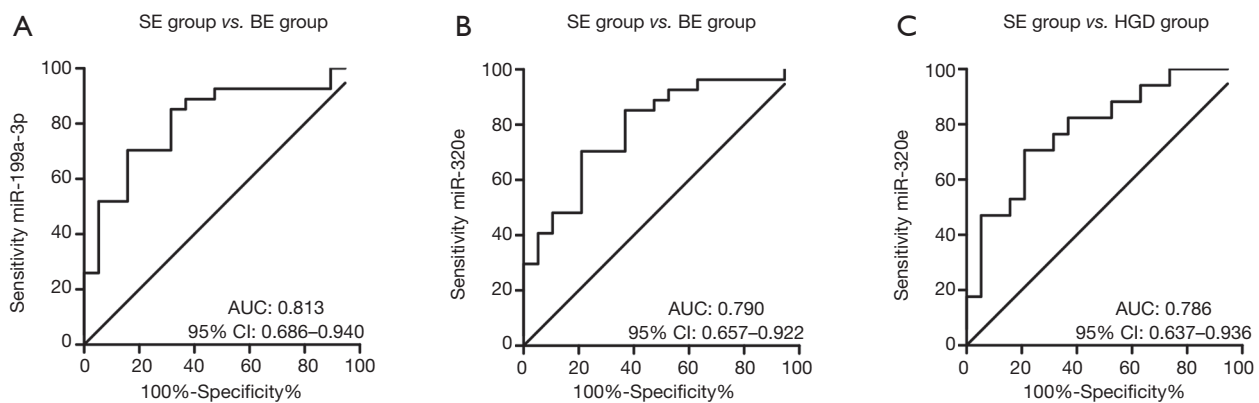


Figure 2 Receiver operating curves (ROC) for miR-199a-3p and miR-320e. (A) Serum miR-199a-3p levels could discriminate between the SE (N=19) and BE (N=27) groups with an area under the curve (AUC) of 0.813 (optimal sensitivity of 85.2% and specificity of 68.4% at relative expression <0.766); (B) serum miR-320e levels could discriminate between SE and BE with an AUC of 0.790 (optimal sensitivity of 85.2% and specificity of 63.2% at relative expression <0.844) and (C) between the SE and HGD (N=17) groups with an AUC of 0.786 (optimal sensitivity of 82.3% and specificity of 62.2% at relative expression <0.838). CI, confidence interval. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia.

not include miR-199a-3p and miR-320e. It is also possible that changes in circulating miRNAs in cancer patients are not only influenced by the tumor itself, but also by systemic processes in the body, such as an inflammatory response or altered metabolism.

The lack of increased levels of any miRNAs during the metaplasia-dysplasia-carcinoma sequence of EAC is surprising, since increased levels of circulating miRNAs have been reported in other solid malignancies (7,9). A single previous study reported increased serum miRNA levels in BE (miR-194-5p and miR-451a) and EAC (miR-451a) (22). In our study, miR-194-5p was not differentially expressed, while miR-451a showed a fold change >2 between the BE and HGD and the HGD and EAC groups. However, no significant differences in miR-451a expression levels were observed in the extended cohort (*Figure S2*). The discrepancy between these findings could reflect differences in specificity of the assays or be due to the limited sample size in our study.

Of the 800 miRNA probes present in the Nanostring system, 62 showed expression levels above the cutoff. This relatively low number can be explained by the low yield of total RNA isolated from serum, the limited number of samples used in the discovery phase and/or the lack of an amplification step. We consider the lack of an amplification step an advantage because it eliminates the risk of selective amplification. However, the combination of a low RNA input in combination with the lack of an amplification step

might have resulted in loss of potentially relevant miRNAs of which the expression levels did not reach the cutoff used in the Nanostring assay.

Finally, PCR validation results were not always consistent with the initial findings from the Nanostring assay; while the trend in expression was similar for miR-150-5p, miR-199a-3p and miR-320e, the fold changes were not statistically significant in the validation cohort. These inconsistencies can possibly be explained by heterogeneity of the patient samples and the limited number of samples used on the Nanostring assay.

Despite the above-mentioned limitations, we feel that the combination of serum samples from all stages of EAC carcinogenesis provides a thorough examination of the miR-199a-3p and miR-320e expression during the metaplasia-dysplasia sequence of EAC carcinogenesis and shown that decreased serum miRNA levels of miR-199a-3p and miR-320e could help to identify patients with BE and HGD, thereby increasing screening efficiency.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

Ethical Statement: The study had been approved by Medische Ethische Toetsingscommissie (METc) from the University Medical Center Groningen, Groningen, The Netherlands (No. 36064.042.11). All participants signed an informed consent form.

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Table S1 Extended patient characteristics						
Age (years)	Study group	Gender	Smoking	Cardiovascular disease	Autoimmune disease	Diabetes
69*	SE	Female	Yes	No	No	No
58*	SE	Male	No	No	No	No
59*	SE	Male	No	No	No	No
52*	SE	Male	Yes	Yes	No	No
61*	SE	Male	No	No	No	No
24*	SE	Male	No	No	No	No
70	SE	Male	No	Yes	No	Yes
53	SE	Female	Yes	No	No	No
53	SE	Female	No	No	No	No
69	SE	Male	No	No	No	No
23	SE	Female	No	No	No	No
65	SE	Female	No	No	No	No
55	SE	Female	No	No	No	No
42	SE	Female	No	No	No	No
47	SE	Male	No	No	No	No
64	SE	Female	No	Yes	No	No
61	SE	Female	No	No	No	No
47	SE	Female	Yes	No	No	No
59	SE	Female	No	No	Yes	No
67*	BE	Male	No	No	No	No
68*	BE	Male	No	No	No	No
67*	BE	Male	No	Yes	No	No
48*	BE	Male	Yes	No	No	No
74*	BE	Male	No	Yes	No	No
69*	BE	Female	Yes	Yes	No	No
57	BE	Female	No	No	No	Yes
63	BE	Female	Yes	No	No	No
67	BE	Female	No	No	No	No
50	BE	Female	No	No	No	No
48	BE	Male	Yes	No	No	No
59	BE	Male	No	No	No	No
42	BE	Female	No	No	No	No
61	BE	Male	No	No	No	No
59	BE	Male	No	No	No	No
70	BE	Male	No	No	No	No
25	BE	Male	No	No	No	No
68	BE	Female	No	No	No	No
25	BE	Male	No	No	No	No
65	BE	Male	No	No	No	No
59	BE	Female	No	No	No	No
60	BE	Male	No	Yes	No	No
67	BE	Male	No	Yes	No	No
75	BE	Male	No	Yes	No	No
55	BE	Male	No	No	No	No
73	BE	Male	No	No	Yes	No
44	BE	Male	Yes	No	No	No
68*	HGD	Male	Yes	No	No	Yes
53*	HGD	Female	Yes	No	No	No
70*	HGD	Male	No	No	No	No
68*	HGD	Male	No	No	No	No
51*	HGD	Male	Yes	No	No	No
69*	HGD	Male	No	No	No	No
74	HGD	Female	No	Yes	No	Yes
62	HGD	Female	No	No	No	Yes
62	HGD	Male	Yes	No	No	Yes
67	HGD	Male	No	Yes	No	Yes
65	HGD	Male	No	No	No	Yes
71	HGD	Male	No	No	No	No
58	HGD	Male	Yes	Yes	Yes	No
42	HGD	Female	No	No	No	No
82	HGD	Male	No	No	No	No
69	HGD	Male	No	Yes	No	No
75	HGD	Male	No	No	No	No
61*	EAC	Male	No	No	No	No
65*	EAC	Female	Yes	No	No	No
73*	EAC	Male	No	No	Yes	No
66*	EAC	Male	No	No	No	No
64*	EAC	Male	Yes	Yes	No	No
46*	EAC	Male	No	Yes	No	No

*, samples used for the Nanostring array. SE, squamous epithelium; BE, Barrett’s metaplasia; HGD, high-grade dysplasia.

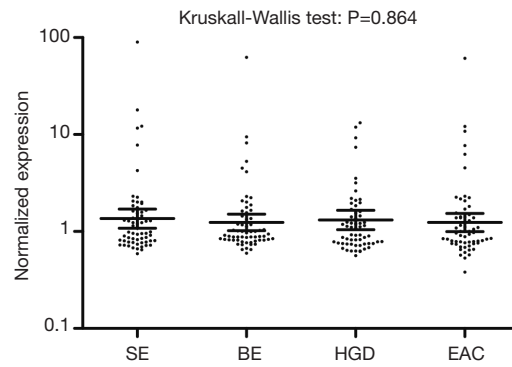


Figure S1 Normalized expression values of 62 serum miRNAs with expression above background in the Nanostring platform. Cutoff of expression was defined as 4 out of 6 of samples in at least one study group having >30 counts. Horizontal lines indicate the geometric mean and the error bars denote the 95% confidence interval. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia; EAC, esophageal adenocarcinoma.

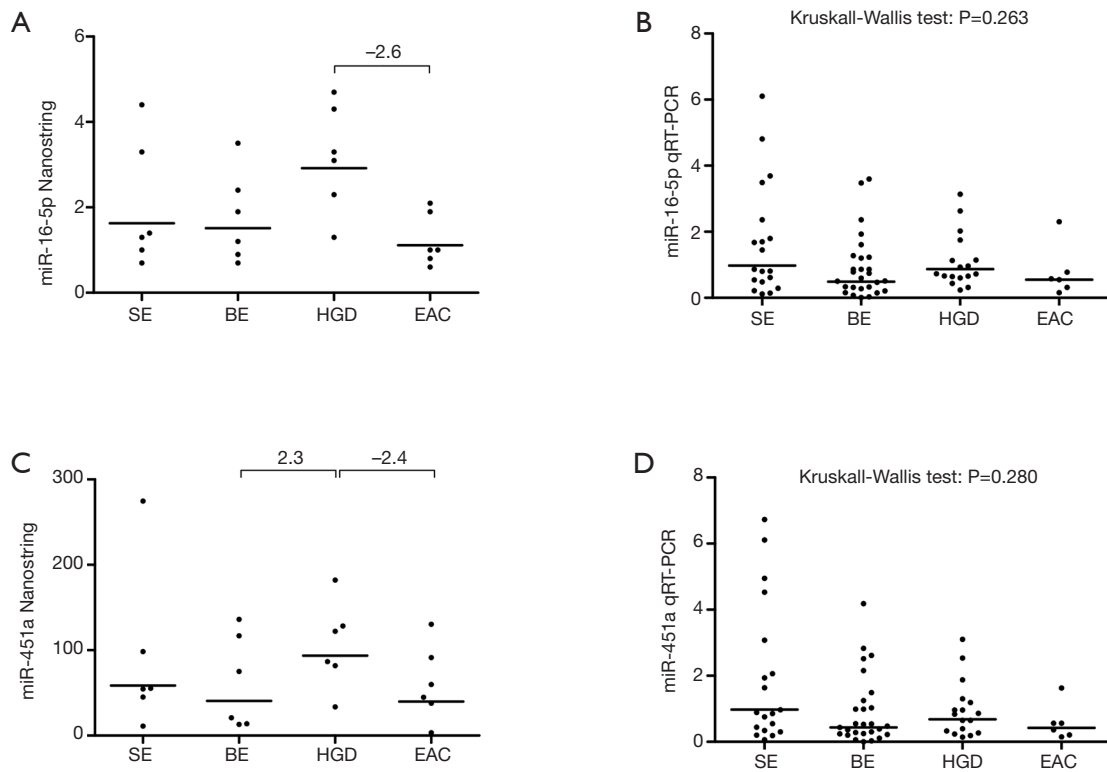


Figure S2 Nanostring and full cohort graphs for miR-16-5p and miR-451a. Y-axis denotes normalized levels on the Nanostring platform (A,C) or RT-qPCR (B,D). Numbers indicate fold changes >2 observed on the Nanostring platform. Horizontal bars denote geometric mean. SE, squamous epithelium; BE, Barrett's metaplasia; HGD, high-grade dysplasia; EAC, esophageal adenocarcinoma.